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<p>(54) Title: <b>METHODS FOR IDENTIFYING FETAL CELLS</b></p> <p>(57) Abstract</p> <p>The invention features a method of distinguishing fetal cells from maternal cells in a sample comprising fetal cells and maternal cells. The method comprises the steps of (a) contacting a sample comprising fetal cells and maternal cells with a probe; the probe being a nucleic acid molecule comprising a nucleotide sequence complementary to the nucleotide sequence of HLA-G mRNA; and, (b) identifying cells in which the probe hybridizes to mRNA in the cells; wherein the absence of hybridization of the probe to mRNA in the cells is an indication that the cells are maternal cells and the presence of hybridization of the probe to mRNA in the cells is an indication that the cells are fetal cells. The invention also features a method of detecting a genetic abnormality in a fetus comprising the steps of (a) contacting a sample comprising fetal cells and maternal cells with a probe; the probe being a nucleic acid molecule comprising a nucleotide sequence complementary to the nucleotide sequence of HLA-G mRNA; (b) isolating cells in which the probe hybridizes to mRNA in the cells, and (c) testing the cells for the presence of the genetic abnormality.</p>		

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## METHODS FOR IDENTIFYING FETAL CELLS

### FIELD OF THE INVENTION

The invention relates to methods of identifying fetal cells in a sample which contains fetal cells and maternal  
5 cells.

### ACKNOWLEDGEMENT OF U.S. GOVERNMENT RIGHTS

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### 10 BACKGROUND OF THE INVENTION

HLA-G, a member of the non-classical HLA class I genes of the human major histocompatibility complex, encodes a nonpolymorphic antigen expressed in placental and some fetal tissues. This fetus-specific histocompatibility gene is  
15 expressed in placental extravillous membranes and in trophoblasts, but has not been found in fetal thymus, skin, and liver. HLA-G specific mRNA has also been found in fetal eye tissues (Shukla et al., *Nucleic Acids Res.* 18:2189, 1990). HLA-G is not expressed in adult T cells, T cell blasts and  
20 several human T and B cell tissue culture lines (Wei et al., *Hum. Immunol.* 29:131-142, 1990). However, expression of this gene has been demonstrated in the human choriocarcinoma cell lines JEG and BeWo (Risk et al., *J.Reprod.Immunol.* 18:199-203, 1990; Ellis et al., *J.Immunol.* 144:731-735, 1990; Ellis et al.,  
25 *Am.J.Reprod.Immunol.* 23:84-86, 1990) and in first trimester chorionic villi (Yelavarthi et al., *J.Immunol.* 146:2847-2854,

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1991; Lata et al., *J.Exp.Med.* 175:1027-1032, 1992). Expression of HLA-G in trophoblasts decreases during the course of pregnancy and is greatly reduced in the third trimester compared with the first trimester (Kovats et al., *Science* 5 248:220-223, 1990). While low levels of an alternatively spliced form of HLA-G have been found in adult lymphocytes (Kirszenbaum et al., 1994, *Proc. Acad. Natl. Sci. USA* 91:4209-4213), expression of HLA-G per se is limited to only a few tissues in the fetus and placenta.

10                   Assessment of fetal development (prenatal diagnosis) is generally conducted by analysis of fetal cells for genetic abnormalities. Procedures for obtaining fetal cells include chorion villus sampling (CVS) and amniocentesis, both of which are termed invasive medical procedures and which have an 15 associated risk of spontaneous miscarriage of less than 1%. There is a long felt need for an non-invasive method of obtaining fetal cells for genetic analysis and for distinguishing these fetal cells from non-fetal cells.

#### **SUMMARY OF THE INVENTION**

20                   The invention features a method of distinguishing fetal cells from maternal cells in a sample comprising fetal cells and maternal cells. The method comprises the steps of (a) contacting a sample comprising fetal cells and maternal cells with a probe; the probe being a nucleic acid molecule 25 comprising a nucleotide sequence complementary to the nucleotide sequence of HLA-G mRNA; and, (b) identifying cells in which the probe hybridizes to mRNA in the cells; wherein the absence of hybridization of the probe to mRNA in the cells is an indication that the cells are maternal cells and the 30 presence of hybridization of the probe to mRNA in the cells is an indication that the cells are fetal cells.

                  In one aspect of the invention, the method comprises isolating the fetal cells from the sample. In another aspect, the sample is maternal blood, mucous from the maternal cervix 35 or mucous from the maternal vagina. Preferably, the sample is maternal blood.

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In yet another aspect of the invention, the method comprises the step of removing non-mononuclear cells from the sample prior to contacting the cells with the probe. In another aspect, maternal cells are depleted from the sample prior to contacting the cells with the probe. In yet another aspect, fetal cells are enriched in the sample prior to contacting the cells with the probe. In addition, in another aspect, maternal cells are depleted from the sample and fetal cells are enriched in the sample prior to contacting the cells with the probe.

According to the methods of the invention, depletion of maternal cells is effected by the steps of incubating the cells in the presence of anti-maternal antibody, further incubation the cells in the presence of anti-anti-maternal antibody, the anti-anti-maternal antibody being conjugated to iron, and removing cells to which the anti-maternal antibody and the anti-anti-maternal antibody are bound using a magnetic separator. In some embodiments, the anti-maternal antibody is murine antibody. Preferably, the anti-maternal antibody is selected from the group consisting of anti-human CD14, anti-human CD4, anti-human CD8, anti-human CD3, anti-human CD19, anti-human CD32, anti-human CD16 and anti-human CD45. Also preferably, the anti-anti-maternal antibody is goat anti-murine antibody.

According to the methods of the invention, enrichment of fetal cells is effected by incubating the cells in the presence of anti-fetal antibody, further incubating the cells in the presence of anti-anti-fetal antibody, the anti-anti-fetal antibody being conjugated to iron, and recovering cells to which the anti-fetal antibody and the anti-anti-fetal antibody are bound using a magnetic separator.

Preferably, the anti-fetal antibody is murine antibody. More preferably, the anti-fetal antibody is selected from the group consisting of anti-human CD71, anti-human CD36 and anti-human glycoporphin A. Also preferably, the anti-anti-fetal antibody is goat anti-mouse antibody.

Further, according to the methods of the invention,

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the probe comprises a portion of the nucleotide sequence complementary to the nucleotide sequence of mRNA encoding HLA-G, wherein said portion hybridizes to said mRNA but does not hybridize to mRNA encoding classical class I HLA antigen.

5           The invention also features a method of detecting a genetic abnormality in a fetus comprising the steps of ( a ) contacting a sample comprising fetal cells and maternal cells with a probe; the probe being a nucleic acid molecule comprising a nucleotide sequence complementary to the  
10 nucleotide sequence of HLA-G mRNA; (b) isolating cells in which the probe hybridizes to mRNA in the cells, and (c) testing the cells for the presence of the genetic abnormality.

          Preferably, the abnormality is selected from the group consisting of trisomy 21, trisomy 13, trisomy 14, trisomy  
15 15, trisomy 16, trisomy 18, trisomy 22, triploidy, tetraploidy, chromosome deletions, sex chromosome abnormalities such as XO, XXY, XYY, XXX, Tay Sachs disease, mucopolysaccharidoses, cystic fibrosis, Duchenne muscular dystrophy, Hemophilia A,  $\beta$ -thalassemia, sickle cell anemia, phenylketonuria and Gaucher  
20 disease.

#### THE DRAWINGS

          Figure 1 is a photograph of an agarose gel depicting electrophoresis of the products of a reverse transcriptase polymerase chain reaction performed on samples of fetal blood.  
25 The source of the blood is indicated at the top of the figure and the size of the DNA products is indicated at the left of the figure. The notation "fb" followed by a series of numbers indicates fetal blood which has been coded for identification. The notation "fb old" is fetal blood which has become degraded.  
30 Jeg-3 is a human choriocarcinoma cell line and is thus derived from fetal cells and the notation "adult" indicates adult blood.

#### DETAILED DESCRIPTION

          The invention features methods of distinguishing  
35 fetal cells from non-fetal cells, particularly maternal cells

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in a sample containing both fetal cells and non-fetal cells. According to the present invention, fetal cells can be identified in and, in some embodiments, isolated from a sample containing maternal cells and fetal cells. The fetal cells, when so identified, may be used in a genetic analysis of the fetus to assess the health of the fetus. In particular, the methods of the invention include the use of HLA-G as a marker to identify fetal cells in a sample containing maternal cells and fetal cells. The methods of the invention may be used as non-invasive methods for identifying fetal cells in samples taken from pregnant women, which methods reduce the risk to both mother and fetus while providing the means to obtain material from the fetus which can be analyzed for genetic defects and/or disorders. The invention is particularly useful in the identification of fetal cells in maternal blood. Thus, the methods of the invention facilitate performance of prenatal diagnosis particularly during the first trimester of pregnancy, and because the methods of the invention rely on tests performed on samples obtained using relatively non-invasive procedures, they are safer for both the mother and fetus than traditional chorion villus sampling and amniocentesis.

The methods of the invention are based on the discovery that expression of HLA-G unambiguously identifies cells of fetal origin in maternal blood and can therefore be used as a marker for fetal cells which are present in low amounts in maternal blood. By "maternal blood" is meant blood obtained from a pregnant mother. It is estimated that nucleated fetal cells are present in maternal blood during the first trimester of pregnancy at a concentration of 1-10 per 10 million maternal mononuclear blood cells. Currently available markers useful for identification of fetal cells in a population of fetal and maternal cells include those capable of identifying male but not female fetal cells, and those which detect chromosome abnormalities present in fetal cells but not maternal cells. Thus, there has been a long felt need for the development of a marker which simply identifies fetal cells in maternal blood, irrespective of gender or the presence or

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absence of chromosomal abnormalities, which cells when so identified, may be examined to determine the health of the developing fetus. Expression of the HLA-G gene provides such a marker. Once a population of cells in maternal blood is  
5 identified as being fetal, techniques and probes for identifying aneuploidy of somatic and sex chromosomes, and for identification of specific genes are readily available in the art.

According to the methods of the invention,  
10 identification of fetal cells is based on expression by the fetal cells of HLA-G. To distinguish fetal cells from maternal cells, expression of HLA-G may be assessed directly in a sample of fetal and maternal cells. In preferred embodiments, maternal cells may be depleted from a sample containing fetal  
15 cells and maternal cells, or fetal cells may be enriched from a sample containing fetal cells and maternal cells, or, both depletion of maternal cells and enrichment of fetal cells may be conducted. Expression of HLA-G in cells of the sample identifies such cells in the sample as being fetal cells. Once  
20 identified, fetal cells may be analyzed in the sample and/or isolated from the sample.

According to the methods of the invention, a sample is provided which contains fetal cells mixed with maternal cells. The sample may be maternal blood, or mucous obtained  
25 from the cervix or vagina of the mother during the early stage of pregnancy. Fetal cells contained in a sample obtained from the cervix or vagina of the mother are likely to be trophoblasts which are shed from the developing placenta. Such trophoblasts can be differentiated from epithelial cells and  
30 maternal leukocytes using techniques known in the art. Preferably, the sample is maternal blood. The amount of maternal blood is preferably 0.01 ml to 50 ml, containing approximately  $10^4$  -  $10^8$  cells. The sample is obtained from the pregnant woman using routine procedures available in the art.

35 In some preferred embodiments, prior to identifying fetal cells in a sample of fetal and maternal cells, the sample is processed to eliminate non-mononucleated cells. Since the



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fetal cells of interest are mononucleated, the elimination of non-mononucleated cells from the sample effectively increases the concentration of fetal cells in the sample. If the sample is a blood sample, elimination of non-mononucleated cells removes maternal red blood cells which comprise the vast majority of the cells in the sample. Methods of isolating mononucleated cells and of elimination of non-mononucleated cells are well known in the art and include, among others, ficoll hypaque centrifugation, or lysis of mature red blood cells using water, bicarbonate or tris-buffered ammonium chloride.

In some preferred embodiments, prior to identifying fetal cells, the maternal cells in the sample are depleted to reduce their number relative to the number of fetal cells. As used herein, the term "maternal cell surface antigens" is meant to refer to cell surface antigens which are found on maternal cells but which are not found on fetal cells or are found infrequently on fetal cells. Examples of maternal cell surface antigens useful in the invention include but are not limited to CD14, CD4, CD8, CD3, CD19 and CD45.

Cells which express maternal cell surface antigens may be selectively removed from a sample using several different techniques known in the art including flow cytometry, affinity chromatography and/or magnetic separation techniques. Examples of methods and apparatuses useful for selectively removing cells which contain maternal cell surface antigens from a sample are described in U.S. Patent Nos. 5,200,084 and 5,186,827, and in Patent Application number WO9415696, each of which is herein incorporated by reference.

According to some embodiments, cells which contain maternal cell surface antigens may be selectively removed using magnetic separation technology. In some embodiments, a sample is first contacted with antibodies which bind to maternal cell surface antigens, also referred to herein as anti-maternal antibodies. Antibodies which bind to anti-maternal antibodies, referred to herein as anti-anti-maternal antibodies are then added. Anti-anti-maternal antibodies are conjugated to iron.

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A magnetic separator is used to isolate cells which have associated with them maternal cell surface antigen. This is accomplished by isolating, from the mixture which includes the sample, cells which have bound to them anti-maternal antibodies  
5 and anti-anti-maternal antibodies conjugated to iron. The isolated cells are discarded and the remaining cells constitute a sample which is depleted of maternal cells. In some embodiments, the anti-maternal antibody is at least one antibody selected from the group consisting of murine anti-  
10 human CD14, CD4, CD8, CD3, CD19, CD32, CD16 and CD45 antibody, and the anti-anti-maternal antibody is a goat anti-murine antibody.

In some embodiments, a single anti-maternal antibody is used to deplete the maternal cells in a sample. In other  
15 embodiments, two or more anti-maternal antibodies are used to deplete the maternal cells in a sample. In embodiments in which two or more anti-maternal antibodies are used to deplete the maternal cells in a sample, the cells are contacted with multiple anti-maternal antibodies in a single step. In other  
20 embodiments in which two or more anti-maternal antibodies are used to deplete the maternal cells in a sample, the cells are contacted with multiple anti-maternal antibodies in sequential steps. In yet other embodiments in which two or more anti-maternal antibodies are used to deplete the maternal cells in  
25 a sample, the cells are contacted with one or more anti-maternal antibodies in a single step, followed by another step in which the cells are further contacted with one or more anti-maternal antibodies.

Selective enrichment of fetal cells in maternal blood  
30 facilitates their identification and isolation. This is accomplished by enriching fetal cells using antibodies to cell surface molecules, which molecules are expressed only on fetal cells, using a variety of magnetic or flow cytometry selection techniques. Techniques and apparatus for separation of cells  
35 using magnetic properties are known in the art and are described in U.S. Patent Nos. 5,200,084 and 5,186,827, and in Patent Application number WO9415696, each of which is herein

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incorporated by reference. The concentration of fetal cells in a sample may be increased relative to the number of maternal cells by selectively removing cells which do not contain cell surface antigens that are found on fetal cells. As used  
5 herein, the term "fetal cell surface antigens" is meant to refer to cell surface antigens that are found on fetal cells. Examples of fetal cell surface antigens include CD71 (transferase receptor protein), CD36 and glycophorin A. CD34, an antigen found only on stem cells, may also be useful in the  
10 methods of the invention.

In some embodiments, a sample is first contacted with antibodies which bind to the fetal cell surface antigens, also referred to herein as anti-fetal antibodies. Antibodies which bind to the anti-fetal antibodies, referred to herein as anti-  
15 anti-fetal antibodies are added. The anti-anti-fetal antibody is conjugated to iron. A magnetic separator is used to isolate cells which have associated with them fetal cell surface antigen. This is accomplished by isolating, from the mixture which includes the sample, cells which have bound to them anti-  
20 fetal antibodies and anti-anti-fetal antibodies conjugated to iron. The isolated cells, constituting a sample enriched for fetal cells, are retained and the remaining cells are discarded. In some embodiments, the anti-fetal antibody is murine anti-human CD71 antibody, murine anti-human CD36 antibody  
25 or murine anti-human glycophorin A antibody and the anti-anti-fetal antibody is goat anti-murine antibody.

In some embodiments of the invention, maternal cells are depleted from the sample prior to identifying fetal cells. In other embodiments, fetal cells are enriched in the sample  
30 prior to identifying fetal cells. In yet other embodiments, maternal cells are depleted from and fetal cells are enriched in the sample prior to identifying fetal cells. In yet other embodiments, fetal cells are identified in the sample in the absence of depletion of maternal cells or enrichment of fetal  
35 cells.

The means for enriching fetal cells in maternal blood are not limited to the methods described above. Rather, other

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means may be used, for example, the methods for isolating fetal cells from maternal blood described in Patent Application No. WO 91/07660 may also be used. These methods include separation of fetal cells from maternal cells using antibodies such as  
5 anti-transferrin antibody and anti HLe-1 (anti-CD45) antibody coupled to cell separation techniques such as cell panning, flow cytometry and/or magnetic separation techniques.

The probes used to identify fetal cells in a sample containing fetal and maternal cells include nucleic acid  
10 molecules which comprise the nucleotide sequence complementary to the nucleotide sequence of mRNA encoding HLA-G, or which comprise a portion of the nucleotide sequence complementary to the nucleotide sequence of mRNA encoding HLA-G, such a portion consisting of a nucleotide sequence sufficient to effect  
15 hybridization of the probe to HLA-G in the absence of cross hybridization of the probe to classical class I HLA antigens. The probe is preferably labelled. In some embodiments, the probe is labelled with a radioactive marker and in other  
20 embodiments, the probe is labelled with a non-radioactive marker, for example, biotin. Preferably, the probe is labelled with biotin and the method for detecting fetal cells in the sample is *in situ* hybridization.

Other probes useful to distinguish, identify, isolate the fetal cells from maternal cells include antibodies specific  
25 for HLA-G. Anti-HLA-G monoclonal antibody is disclosed in Odum et al. (1991, Eur. J. Immunol. 21:2121-2131).

The means for detecting expression of HLA-G in fetal cells are not limited to the use of biotinylated probes in *in situ* hybridization. Other methods for detection of expression  
30 of HLA-G in fetal cells may also be used. These methods include use of an HLA-G probe which is radiolabelled, or which is labelled with a non-radioactive compound other than biotin in *in situ* hybridization experiments. Fetal cells may also be identified through the use of *in situ* PCR technology using  
35 primers specific for amplification of HLA-G specific mRNA. Nucleic acid may be extracted from fetal cells and Northern blot hybridization or PCR may be performed to identify HLA-G

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specific mRNA. HLA-G specific antibodies may also be used to identify fetal cells expressing HLA-G either within or on the surface of the cells. Further, an HLA-G probe which is smaller than the full length of the cDNA encoding HLA-G may be used  
5 provided that such a probe does not appreciably cross-hybridize with other HLA-specific nucleic acids thereby resulting in false positive identification of fetal cells.

HLA-G serves as an independent marker for fetal nucleated cells and thus facilitates their differentiation from  
10 adult cells in maternal blood. Since present technology for identification of fetal cells in a population of adult cells relies on the demonstration of male cells in female blood or the presence of fetal or embryonic hemoglobins that are not expressed by adult cells, use of the methods of the invention  
15 employing HLA-G provides significant advantages over present methods. Further, although antibodies which react with trophoblasts have been reported, many such antibodies cross react with adult (maternal) cells and therefore do not provide reliable identification of fetal cells in maternal blood.  
20 Since examination of fetal cells by chorion villus sampling or amniocentesis incurs the risk of spontaneous miscarriage, use of HLA-G as a marker for fetal cells in maternal blood provides a relatively non-invasive procedure for accurate assessment of the health of the developing fetus. Fetal cells so identified  
25 may then be tested, using procedures known in the art, to determine whether or not they exhibit characteristics of any one of a variety of disease states.

In addition to serving as an independent fetal cell marker, reagents which specifically react with HLA-G, including  
30 cDNA probes or antibodies to unique cell surface determinants encoded by the HLA-G gene, are useful as tools for purifying fetal cells from maternal blood. For example, a population of cells may be incubated in the presence of a biotinylated cDNA probe specific for HLA-G mRNA. Cells may be further incubated  
35 in the presence of avidin conjugated to iron or anti-biotin antibody conjugated to iron. Cells to which the probe has bound will also bind avidin or anti-biotin antibody and may be

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thus identified and/or separated from the population using a magnetic separator.

In addition, reverse-transcriptase polymerase chain reaction (RT-PCR) specific for HLA-G mRNA could be used to  
5 quantitate fetal cells in maternal blood. Quantitative reverse transcriptase-PCR is known in the art. The number of fetal cells in a population of cells whose total number is known may be estimated by comparing the quantity of mRNA specific for HLA-G in the cell population to the quantity of mRNA specific  
10 for classical class I HLA antigens in same cell population. Assuming that each cell expresses approximately the same amount of classical class I HLA specific mRNA, the number of fetal cells in the population may be estimated from the amount of HLA-G specific mRNA present in the population. Diseases which  
15 can be detected in the fetus, by examination of fetal cells obtained following the methods of the invention, include all diseases for which there is a characteristic karyotype, including diseases associated with aneuploidy such as Down Syndrome (trisomy 21) and other trisomies (usually 13, 14, 15,  
20 16, 18, or 22). Other diseases include those associated with the presence of extra sets of chromosomes (triploidy or tetraploidy), those associated with deletions of part of a chromosome, those associated with sex chromosome abnormalities (XO, XXY, XYY, XXX), and those associated with balanced and  
25 unbalanced rearrangements of chromosomes, such as translocations. Diseases which are associated with chromosome breaks include Fanconi's anemia, Bloom's syndrome and Ataxia telangiectasia. Diseases which may also be detected in the fetus include those which can be diagnosed by protein/enzyme  
30 abnormalities such as but not limited to, Tay Sachs disease and the mucopolysaccharidoses. In addition, single gene disorders may be diagnosed. Upon identification of the gene in question, restriction fragment length polymorphism analysis or PCR analysis can be conducted to diagnose cystic fibrosis, Duchenne  
35 muscular dystrophy, Hemophilia A,  $\beta$ -thalassemia, sickle cell anemia, phenylketonuria, Gaucher disease, Severe Combined Immunodeficiency Disorder, and any other disease for which a

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probe, an enzyme or a cell surface marker is known. All of the above named conditions and the associated chromosome or enzyme abnormalities are known in the art as are methods of detecting these diseases. In fact, the methods of the invention may be used to detect any disease in a fetus for which a gene, a probe, a chromosome abnormality or an enzyme abnormality is known.

The following provides some examples of the present invention. These examples are not to be considered as limiting the scope of the appended claims.

#### EXAMPLES

**Example 1.** To effect isolation of fetal cells in maternal blood, maternal cells are first depleted from the sample. This is accomplished by separating mononuclear cells (MNCs) from maternal peripheral venous blood (usually 20 ml) by standard ficoll hypaque centrifugation. Maternal MNC are then depleted from the sample by first adding to the sample of cells a series of mouse anti-human antibodies. Antibodies typically employed are commercially available and may include those to CD14 (monocytes), CD4 and CD8 (T cells), CD3 (T cells), CD19 (B cells), CD32 and CD16 (granulocytes) and CD45 (leukocytes in general). Next, goat anti-mouse antibodies are added to the sample, which antibodies are conjugated to iron (Ferrofluid, Immunicon). Cells with bound antibodies are separated from cells without bound antibodies using a magnetic cell separator (Immunicon Corp.). Those cells with antibodies bound to them will be retained on magnetized wire loops or surfaces in the magnetic cell separator. This procedure, results in depletion of as much as 99% or more of adult MNC.

In general, either separately or sequentially, mononuclear cells are incubated with 20  $\mu$ l of commercially available monoclonal antibody for 30-45 minutes on ice. The cells are then reincubated with Ferrofluid (the stock solution is diluted 1:40-1:100 in physiological solution), washed, and then "pulled" to wire loops which have been magnetized in a device which generates a high gradient magnetic field

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(Immunicon magnetic cell separator). The monoclonal antibodies are used as prepared by the suppliers. The combination of antibodies used is preferably CD14 and CD45, or CD14, CD4, CD8, CD32, CD16 and CD19.

5                   When depletion of maternal cells is complete, positive selection of fetal cells in the sample is accomplished using a magnetic cell separator, ferrofluid and antibodies to CD71, CD36 or glycophorin A. CD71 is the transferrin receptor which is expressed abundantly on fetal nucleated red blood  
10 cells. Generally, cells remaining following depletion are incubated in the presence of 20  $\mu$ l of anti-CD71 or anti-glycophorin A (a red cell marker expressed to high levels on fetal erythrocytes and their precursor cells) or with a combination of both antibodies. Following 30-45 minutes on ice  
15 , Ferrofluid is added and the cells are positively selected onto magnetized wire loops. These cells are washed in the magnetic field and then are recovered from the wire loops following removal of the magnetic field.

20                   Messenger RNA specific for HLA-G is detected by *in situ* cDNA-mRNA hybridization following published methodology (Lo, *J.Cell.Sci.* 81:143-162, 1986; Tuan et al., *Mol.Biochem.Parasit.* 49:191-204, 1991; Haynes et al., *Cell.Immunol.* 151:300-308, 1993). A cDNA probe specific for  
25 HLA-G useful for demonstrating expression of HLA-G in first trimester trophoblast is used (Lata et al., *J.Exp.Med.* 175:1027-1032, 1992). Essentially, target tissue is incubated in the presence of biotinylated cDNA probe under conditions suitable for hybridization of the probe to HLA-G mRNA. The  
30 sequence of the HLA-G cDNA probe is disclosed in Geraghty et al. (1987, *Proc. Natl. Acad. Sci. USA* 84:9145-9149) which is herein incorporated by reference. The hybridized probe is then visualized by reaction with streptavidin-alkaline phosphatase and chromogenic substrate. Examination of nucleated cells in  
35 fetal peripheral blood revealed that HLA-G mRNA is present in 100% of these cells at 10-12 weeks gestation and that the percentage of HLA-G positive cells decreased to about 60% by



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20 weeks gestation. These results are presented below.

Table 1

12 Week Fetal Blood		
Marker	Fetal Blood MNC	Adult Peripheral Blood MNC
*Bluescript	Neg.	Neg. (Few Cells)
HLA-Class I (B7)	Neg.	Str. Pos. (All cells)
$\beta$ -2 Microglobulin	Neg. (Occas. Wk. +)	Pos. (Few Cells)
HLA-DRB	Neg.	No Cells Seen
HLA-G	Str. Pos. (All Cells)	Neg.
20 Week Fetal Blood		
Marker	Fetal Blood MNC	Adult Peripheral Blood MNC
*Bluescript	Neg.	Neg.
HLA-Class I (B7)	25% Positive	100% Positive
HLA-G	60% Positive	Neg.

15 \*Bluescript represents the control probe used.

These data suggest a possible inverse relationship between expression of classical MHC genes and HLA-G. Cells positive for HLA-G mRNA were also found in blood obtained from the intervillous space.

20 **Example 2.** To determine which types of fetal peripheral blood cells might enter the intervillous space and the maternal circulation, flow cytometry was performed on samples of 12 week fetal blood. In two separate experiments greater than 90% of fetal nucleated peripheral blood cells expressed high levels of transferrin receptor (CD71) and less than 5% of these cells were positive for the general leukocyte marker CD45. The latter result is clone dependent, in that up to 20% of fetal cells reacted with clone J.33 (Beckton-

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Dickinson) and clone ALB-12 (Amac) providing data indicating the <5% are positive for CD45 (the CD45 molecule has a number of distinct antigenic epitopes and the antibodies to these epitopes are not necessarily cross-reactive).

5           These data are consistent with the results of experiments determining the phenotypes of cells in matched intervillous space (IVS) and maternal peripheral blood. The results of two such experiments in which limited phenotyping was completed on matched samples are given below.

10   **Table 2: Phenotypes of IVS and peripheral blood mononuclear cells by flow cytometry\*.**

Marker**	Experiment 1		Experiment 2	
	IVS	Blood	IVS	Blood
CD2	17.41%	70.08%	16.30%	77.30%
15   TCR $\alpha\beta$	16.97	66.63	13.22	71.88
TCR $\gamma\delta$	0.86	2.30	0.08	1.97
GLY-A	ND	ND	72.00	2.70
CD56*16*	0.74	12.61	2.65	16.62
CD8	10.44	19.79	6.03	34.37
20   CD4	6.87	50.49	6.49	38.36

\* Gates were set on the CD45+ population of leukocytes in each sample.

\* Data are presented as % of gated cells.

\*\* TCR=T cell receptor

25           Since there are clear physiologic or mechanical reasons why fetal blood cells enter the maternal circulation during normal pregnancy (Bianchi, D.W., et al., *Proc.Natl.Acad.Sci. USA* 87:3279-3283, 1990, Wachtel, S., et al., *Hum.Reprod.* 6:1466-1469, 1991, Price, et al., *Am.J.Obstet.Gynecol.* 165:1731-1737, 1991), it is likely that these  
 30 results reflect natural movement of fetal cells into the maternal blood during pregnancy rather than movement caused by bleeding into the IVS from severed vessels in the villi during the experimental procedure.

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**Example 3.** To identify fetal cells using RT-PCR, first trimester fetal blood was isolated during fetal reduction surgery. Messenger RNA was then isolated directly from fetal blood so obtained by adding 0.4 ml of a guanidinium thiocyanate/N-lauroyl sarcosine-based extraction buffer (Quikprep mRNA isolation kit, Pharmacia) to an equal volume of blood. The solution was vortexed, diluted in two volumes of Tris-EDTA (pH 8.0), centrifuged at 12000 rpm for 4 minutes in a microfuge and 25 mg of oligo(dT)-cellulose was added to the resulting supernatant. The mixture was incubated on a shaking platform for 30 minutes, centrifuged at 12000 rpm for 15 seconds and the cellulose containing bound mRNA was washed successively in high salt (0.5 M NaCl/Tris-EDTA) and low salt (0.1 M NaCl/Tris-EDTA) buffers. Bound mRNA was eluted from oligo(dT)-cellulose using 2 bed volumes of pre-warmed Tris-EDTA (pre-warmed to 65°C) and was then ethanol precipitated in the presence of glycogen and potassium acetate. The mRNA was resuspended in 0.1% Tris-EDTA and was stored at -80°C until used in RT-PCR analysis.

To perform RT-PCR, first, cDNA synthesis was conducted at 37°C for 45 minutes using 10 µl of mRNA template in a reaction mixture containing 400 units of M-MLV reverse transcriptase (Gibco-BRL), 5 mM dithiothreitol, 0.25 mM of each of the four dNTPs, 40 units of RNase inhibitor (Promega) and random hexamers (5 µM, Perkin Elmer) in a 40 µl volume of RT buffer (Gibco-BRL). Next, amplification of cDNA was accomplished by incubating 5 µl of the RT reaction mixture with 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), 0.2 mM of each of four dNTPs, 1.5 mM MgCl<sub>2</sub> and 50 ng of the HLA-G sense and antisense primers (Shukla et al., *Nucleic Acids Res.* 18:2189, 1990). These primers are as follows:

Sense: 5'-TTGTCCTTGCAGCTGTAG-3'

Antisense: 5'-CTGGTCTCTGCACAAAGAGAAG-3'.

Samples were hot-started by incubation at 95°C for 4 minutes prior to the addition of Taq polymerase. Reactions were carried out for 35 cycles using a model 50/60 tempcycler (Coy Laboratory Products) with cycling parameters of 95°C for 45

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seconds, 52°C for 45 seconds and 72°C for 60 seconds. The results of this experiment are presented in Figure 1. Eight fetal blood samples were tested and of these, six expressed the expected 185 base pair fragment indicating that HLA-G mRNA is present in fetal blood in the first trimester. This 185 base pair fragment was also observed in JEG cells (positive control) and was absent in a sample obtained from adult blood (negative control). These results establish that fetal blood cells may be identified using HLA-G specific probes is an RT-PCR reaction.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

- 19 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Thomas Jefferson University, et al.

(ii) TITLE OF INVENTION: Methods For Identifying Fetal Cells

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

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## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PC Compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: WORDPERFECT 5.1

## (vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/347, 506

(B) FILING DATE: 30-NOV-1994

## (vii) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Not Yet Known

(B) FILING DATE: Herewith

(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

- 20 -

(A) NAME: Leary, Kathyrn

(B) REGISTRATION NUMBER: 36,317

(C) REFERENCE/DOCKET NUMBER: TJU-1703

## (ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (215) 568-3439

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTGTCCTTGC AGCTGTAG

18

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTGGTCTCTG CACAAAGAGA AG

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- 21 -

What is claimed is:

1. A method of distinguishing fetal cells from maternal cells in a sample comprising fetal cells and maternal cells, said method comprising
  - 5 (a) contacting a sample comprising fetal cells and maternal cells with a probe; said probe being a nucleic acid molecule comprising a nucleotide sequence complementary to the nucleotide sequence of HLA-G mRNA; and,
  - (b) identifying cells in which said probe hybridizes  
10 to mRNA in said cells; wherein the absence of hybridization of said probe to mRNA in said cells is an indication that said cells are maternal cells and the presence of hybridization of said probe to mRNA in said cells is an indication that said cells are fetal cells.
- 15 2. The method of claim 1 further comprising isolating said fetal cells from said sample.
3. The method of claim 1, wherein said sample is maternal blood, mucous from the maternal cervix or mucous from the maternal vagina.
- 20 4. The method of claim 3, wherein said sample is maternal blood.
5. The method of claim 1 further comprising the step of removing non-mononuclear cells from said sample prior to contacting said cells with said probe.
- 25 6. The method of claim 1, wherein maternal cells are depleted from said sample prior to contacting said cells with said probe.
7. The method of claim 1, wherein fetal cells are enriched in said sample prior to contacting said cells with  
30 said probe.

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8. The method of claim 5, wherein maternal cells are depleted from said sample and fetal cells are enriched in said sample prior to contacting said cells with said probe.

9. The method of claim 6, wherein said depletion of maternal cells is effected by the steps of incubating said cells in the presence of anti-maternal antibody,

further incubation said cells in the presence of anti-anti-maternal antibody, wherein said anti-anti-maternal antibody is conjugated to iron, and

removing cells to which said anti-maternal antibody and said anti-anti-maternal antibody are bound using a magnetic separator.

10. The method of claim 9, wherein said anti-maternal antibody is murine antibody.

11. The method of claim 10, wherein said murine antibody is selected from the group consisting of anti-human CD14, anti-human CD4, anti-human CD8, anti-human CD3, anti-human CD19, human anti-CD32, human anti-CD16 and anti-human CD45.

12. The method of claim 9, wherein said anti-anti-maternal antibody is goat anti-murine antibody.

13. The method of claim 7, wherein said enrichment of said fetal cells is effected by incubating said cells in the presence of anti-fetal antibody,

further incubating said cells in the presence of anti-anti-fetal antibody, wherein said anti-anti-fetal antibody is conjugated to iron, and

recovering cells to which said anti-fetal antibody and said anti-anti-fetal antibody are bound using a magnetic separator.



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14. The method of claim 13, wherein said anti-fetal antibody is murine antibody.

15. The method of claim 13 wherein said murine antibody is selected from the group consisting of anti-human CD71, anti-human CD36 and anti-human glycophorin A.

16. The method of claim 13, wherein said anti-anti-fetal antibody is goat anti-mouse antibody.

17. The method of claim 1, wherein said probe comprises a portion of the nucleotide sequence complementary to the nucleotide sequence of mRNA encoding HLA-G, wherein said portion hybridizes to said mRNA but does not hybridize to mRNA encoding classical class I HLA antigen.

18. A method of detecting a genetic abnormality in a fetus comprising

15 (a) contacting a sample comprising fetal cells and maternal cells with a probe; said probe being a nucleic acid molecule comprising a nucleotide sequence complementary to the nucleotide sequence of HLA-G mRNA;

(b) isolating cells in which said probe hybridizes

20 to mRNA in said cells, and

(c) testing said cells for the presence of said genetic abnormality.

19. The method of claim 18, wherein said abnormality is selected from the group consisting of trisomy 25 21, trisomy 13, trisomy 14, trisomy 15, trisomy 16, trisomy 18, trisomy 22, triploidy, tetraploidy, chromosome deletions, chromosome translocations, chromosome breaks, sex chromosome abnormalities, Tay Sachs disease, mucopolysaccharidoses, cystic fibrosis, Duchenne muscular dystrophy, Hemophilia A, 30  $\beta$ -thalassemia, sickle cell anemia, phenylketonuria, Gaucher disease, Fanconi's anemia, Bloom's syndrome, Severe Combined Immunodeficiency Disorder and Ataxia telangiectasia.

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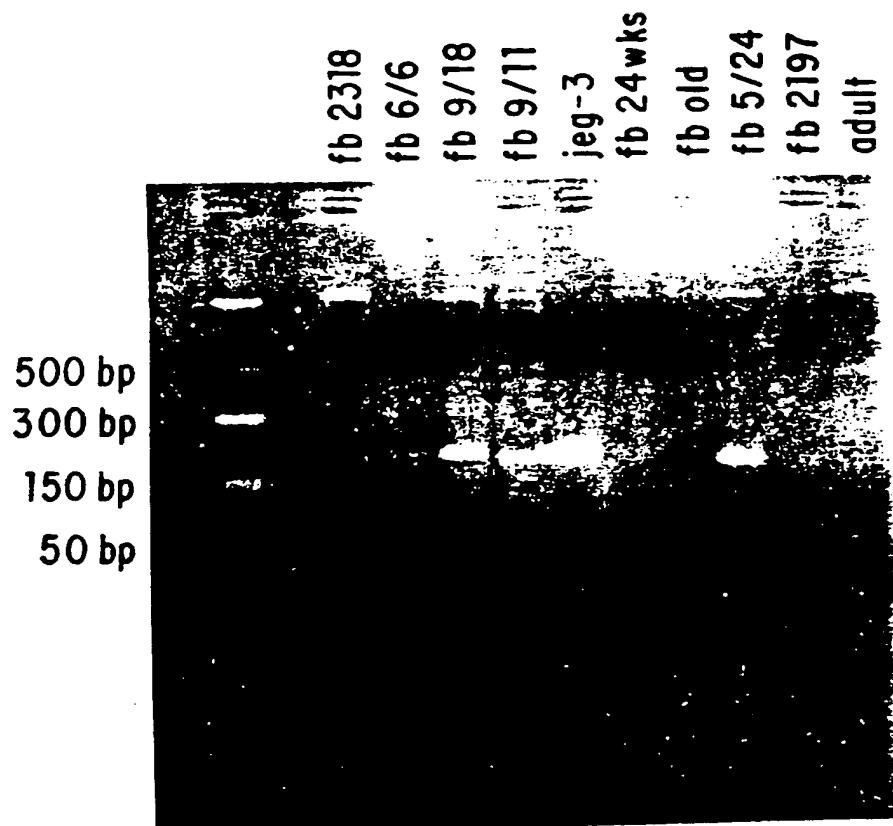


FIG. 1

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/15444

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68

US CL :435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,153,117 (SIMONS) 06 October 1992, Abstract, columns 2 -3, 8-9.	1-19
A	US, A, 5,200,084 (LIBERTI ET AL) 06 April 1993.	1-19
P,Y	The Journal of Histochemistry and Cytochemistry, Vol. 43, No. 7, issued July 1995, Van Wijk et al., "Multiparameter in situ analysis of trophoblast cells in mixed cell populations by combined DNA and RNA in situ hybridization", pages 709-714, see entire document.	1-19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 MARCH 1996

Date of mailing of the international search report

25 MAR 1996

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## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US95/15444

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	American Journal of Reproductive Immunology, Vol. 30, No. 2-3, issued 1993, Ganshirt-Ahlert et al, "Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting", pages 194-201, see pages 194-195 and 198.	1-8, 13-19
A	Proceedings of the National Academy of Science, USA, Vol. 91, issued May 1994, Kirszenbaum et al, "An alternatively spliced form of HLA-G mRNA in human trophoblasts and evidence for the presence of HLA-G transcript in adult lymphocytes", pages 4209-4213, see entire document.	1-19
P, Y	Prenatal Diagnosis, Vol. 14, issued December 1994, Busch et al, "Enrichment of fetal cells from maternal blood by high gradient magnetic cell sorting (double MACS) for PCR-based genetic analysis", pages 1129-1140, see pages 1129-1130, 1132-1133 and 1136-1137.	1-17
A	Journal of Experimental Medicine, Vol. 175, issued April 1992, Lata et al., "Localization of major histocompatibility complex class I and II mRNA in human first-trimester chorionic villi by in situ hybridization", pages 1027-1032, see pages 1027 and 1030-1031.	1-19
Y	Human Reproduction, Vol. 9, No. 6, issued June 1994, Yagel et al, "Trophoblasts circulating in maternal blood as candidates for prenatal genetic evaluation", pages 1184-1189, see entire document.	1-19
A	Nucleic Acids Research, Vol. 18, No. 8, issued 25 April 1990, Shukla et al, "The mRNA of a human class I gene HLA G/HLA 6.0 exhibits a restricted pattern of expression", page 2189.	1-19

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/15444

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, EMBASE

search terms: pre-natal, prenatal, test###, diagnosis, magnetic, HLA-G, fetal, foetal, restricted, specific, CD14, CD4, CD8, CD3, CD19, CD32, CD16, CD45, CD71, CD36, glycophorin, mRNA, cDNA

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